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(54)   Title: TGC METHOD FOR INDUCTION OF TARGETED SOMATIC TRANSGENESIS

(57)   Abstract: [in English]

TGC Method for Induction of Targeted Somatic Transgenesis

The subject of the invention is a method for induction of a targeted somatic transgenesis (TGC = targeted genetic conditioning), which is used for the expression of foreign proteins in cells, a tissue, an organ or an entire host organism and for somatic gene therapy.

It is known that proteins can be expressed in sufficient quantities for technical applications or for therapeutic purposes by the transfer of genes into microorganisms or mammalian cells. These methods are especially important for the body's own proteins, which are otherwise not accessible or only slightly accessible, such as hormones, regulation factors, enzymes, enzyme inhibitors and humanized monoclonal antibodies, as well as for the production of surface proteins of pathogenic microorganisms or viral envelope proteins for the safe production of diagnostic tests and tolerated vaccines. "Protein engineering" can also produce novel proteins which obtain application-optimized properties by fusion, mutation, or deletion of appropriate DNA sequences, such as immunotoxins.

Genes obtained from human cells are also able to function in mouse, rat or sheep cells and lead to the formation of corresponding gene products there. This has already been employed in practice for the production of therapeutic proteins, for example, in the milk of transgenic farm animals. The hitherto known method for this is microinjection of corresponding vectors carrying foreign DNA into the nucleus of the fertilized egg cell, where the DNA is then incorporated into the chromosome at a yield of 1%. The transgenic fertilized egg cell is then reimplanted in hormonally stimulated dams. A progeny which carries the introduced gene in all somatic cells is the foundation for the creation of a "transgenic flock." Thanks to the use of gene technology it has also become possible to specifically alter farm animals so that they produce human proteins in their blood, their tissue, or their milk, such as cannot be produced in microorganisms or plants.

However, the use of transgenic animals as protein production factories has the major drawback of requiring an intervention in the germ tract of the animals. Owing to the high technical and time outlay for the creation and raising of transgenic animals and also because of the debate on the ethical consequences of these methods, alternative methods for protein production in an animal host without germ tract intervention are necessary and would be of very great benefit.

It is furthermore known that the milk of mammals such as cows, sheep, goats, horses or pigs can contain a number of bacterial disease pathogens. Among them are Listeria, Mycobacteria, Brucella, Rhodococcus, Salmonella, Shigella, Escherichia, Aeromonades and Yersinia, or generally bacteria with an intracellular lifestyle [1, 2]. These bacteria are essentially transferred to man or animal by oral ingestion [3], but droplet infections also play a role. A primary source for human infection with Listeria [4], Mycobacteria [5] and Escherichia coli is contaminated milk [6]. The person takes up the bacteria by consuming nonpasteurized milk or milk products. The other bacterial genera mentioned above, such as Salmonella, Shigella, Yersinia, Rhodococcus and Brucella, are transmitted in similar fashion to man. But bacteria can also get into man by other animal products from the cow, the goat, the sheep, the rabbit, the horse, the pig or poultry that are affected with bacteria.

The infection of animals often occurs through mucosal surfaces, very often through the digestive tract. However, not all tissues are symptomatically infected after taking up the bacteria, as in the case of Listeria. In the cow and in the goat, the infection of udders, spleen and liver are in the foreground of the infection process. In sheep, moreover, the central nervous system can be attacked in the form of meningitis, so that not all animals survive the infection. Infection of the udders closes the chain of the infection. Bacteria taken up with contaminated milk can then reinfect the animal, for example, the suckling calf, or humans through the digestive tract.

At present, the following is known as to the route of bacterial infection of man, especially presented here by the example of the Listeria:

Of the six known Listeria species, only L. monocytogenes and L. ivanovii [7] are pathogenic to man. The human disease occurs when infected milk or milk products are consumed. The course of the infection depends on the state of health of the person and is generally nondramatic. During pregnancy, an intrauterine transmission of the germ to the fetus can occur, associated with miscarriage, still birth, or premature birth. In all cases, an excellent and problem-free treatment with antibiotics such as Ampicillin or Erythromycin is possible [8, 8a].

For L. monocytogenes in man and animal, and for L. ivanovii in the sheep, the way into the cell is well defined. A number of pathogenicity factors are needed for Listeria to have full pathogenicity. They include PrfA (positive regulator of virulence), ActA (actin nucleating protein), PlcA (phosphatidylinositol-specific phospholipase), PlcB (phosphatidylcholine-specific phospholipase), Hly (listeriolysin), Mpl (metalloprotease) [9]. The cell specificity of the pathogen/host cell interaction is mediated through a series of proteins. They include the internalins InlA and InlB, which are involved in the initial contact and the interaction between bacteria and cell surface [10, 11]. All monocytogenes in experimental situations can infect endothelial cells, epithelia cells, fibroblasts and hepatocytes, among others. Moreover, L. monocytogenes also infects cells of the white blood picture with neutrophilic granulocytes, macrophages and lymphocytes. This is a major factor in the transmission of the bacteria from the entry portal to the target organ in the host. Finally, the lung tissue can also be infected by Listeria, if the bacteria are applied via droplet infection.

After adhesion to the cell surface, L. monocytogenes is ushered into the cell by endocytosis, the endosome membrane is destroyed by the action of listeriolysin (Hly) and thus it is released into the cell's cytosol [14]. Once arrived in the cell, the microbe can multiply. During the production of additional proteins, the fully pathogenic bacterium does not remain stationary, but actively propagates itself. The propagation is accomplished by the use of a series of proteins peculiar to L. monocytogenes and some of the cell's own proteins [15, 16]. ActA is expressed on the cell surface of L. monocytogenes. It binds the cellular protein VASP, which in turn forms the bridge for attachment of cellular actin. In the subsequent course, actin tails are formed, which carry the bacterium at the tip and thus propagate it through the cell. If L. monocytogenes encounters the cell membrane, a membrane protrusion is formed, which penetrates directly into the neighboring cell when such are present. This protuberance is then endocytized by the neighboring cell, so that L. monocytogenes finds itself inside a double membrane in the new cell. The two membranes are dissolved under the action of Hly and PlcB [17]. At the end of this process, L. monocytogenes has also infected the neighboring cell and the infection process starts over again. In this way, L.

monocytogenes gets into the secretory cells of the cow udder. Secreted Listeria proteins can be detected in the milk, i.e., they are put out intracellularly from the lactating cell into the milk [18]. These proteins include Hly (listeriolysin) and IrpA (internalin related protein [19]), two pathogenicity factors which are produced in great quantities by L. monocytogenes in the host, secreted, and eliminated in the milk [20].

This knowledge about the infection process has made it possible to genetically alter L. monocytogenes so that it expressed foreign proteins. Examples of the expression of foreign proteins in L. monocytogenes are: alkaline phosphatase from Escherichia coli, the nucleoprotein from the lymphocoriomeningitis virus (LCMV), the nucleoprotein from the influenza virus, the "major capsid protein" (L1) from cottontail rabbit papilloma virus (CRPV), and the gag protein from HIV Type 1 [20 to 27].

Besides proteins of procaryotic origin, there are also viral proteins not normally produced within the eukaryotic cell. These and similar foreign proteins of procaryotic and eukaryotic origin can be produced by L. monocytogenes without requiring a eukaryotic cell for this. Proteins produced by L. monocytogenes are eliminated in the milk.

The infection by bacteria occurs through specific interactions of ligands/proteins of the bacteria with receptor proteins of the target cells. In the case of L. monocytogenes, the internalin family is critically involved in this; it essentially determines the cell specificity of the infection process [28]. Moreover, an ActA-dependent cell uptake is contemplated, which is mediated by receptors of the heparane sulfate family [29]. If L. monocytogenes infects the cell, a full infection cycle does not occur in every case. If listeriolysin is switched off in L. monocytogenes, the bacteria remain stuck in the endosome and the infection of the "first cell" does not come to pass. Bacteria in which the protein ActA is switched off, inactive, or no longer present enter the first-infected cells, but remain stuck there and can no longer infect the neighboring cells [30, 31]. When Pc1B is switched off, the microbe is no longer able to establish itself in a second cell.

L. monocytogenes is a bacterium which can be treated by a series of antibiotics. Especially suitable are ampicillin and penicillin (each in combination with gentamycin). As alternatives, erythromycin and sulfonamides are also used. In special cases, tetracyclins, vancomycin or chloramphenicol are employed [32]. Corresponding treatment possibilities also exist for other bacteria [8a] of the genera Aeromonas, Bartonella, Brucella, Campylobacter, Enterobacteriaceae, Mycobacterium, Renibacterium, Rhodococcus or other bacteria that are genetically or biochemically related to the mentioned bacterium. In consideration of this knowledge, the problem now arose of using a bacterial infection for a method of organotropic protein production.

This problem is solved by a TGC method for induction of a targeted, somatic transgenesis, in which bacteria which carry a foreign DNA, integrated in an episomal vector and prepared for subsequent transcription and expression, upon infection of cells, tissues, an organ or the entire host organism release their genetic information into the individual infected cell and thereby accomplish the expression of foreign protein.

This method can be used either to harvest a foreign protein, or also advantageously for somatic gene therapy, in which the foreign DNA introduced by the bacterial infection into the host organism

brings about there the formation of a protein lacking in the host organism or increases, decreases or prevents the formation of a protein in the host organism by the production of single or double-stranded nucleic acid. This method can be applied to all known farm animals and also to man.

If the infected tissue is the egg of an avian, the foreign protein will be produced in the egg and can be isolated from it by the familiar techniques of protein isolation, for example, from chicken eggs. If the infected cells are blood cells, a propagation of the bacteria and with them the foreign DNA in the entire injected [sic!] organism can be achieved by parenteral infection of the cells. If the host animals are experimental animals, whose infected organ is an udder, the desired foreign protein will be produced in the milk of the experimental animal, and the foreign protein can then be isolated from it.

The TGC method shall be discussed hereafter on the example of the bacterium L. monocytogenes. However, it can also be applied accordingly in all intracellularly growing bacteria, especially the bacteria of the genera Aeromonas, Bartonella, Brucella, Campylobacter, Clostridia, Enterobacteriaceae (especially in the latter bacteria of the species Yersinia, Escherichia, Shigella, Salmonella), Legionella, Mycobacterium, Renibacterium, Rhodococcus or bacteria from genera genetically or biochemically related to them, although other bacterial genera that are not pathogenic and also do not have any intracellular life cycle are also suitable for the method of the invention, as long as they are viable in an eukaryotic host organism.

Furthermore, it is possible to carry out the TGC method with originally apathogenic bacteria that are outfitted by genetic manipulation with additional factors allowing them to enter into the cell. Many bacteria not growing intracellularly by nature can be used for such an "outfitting with pathogenicity factors," such as Bacillus subtilis, Lactobacilli, Pseudomonades, Staphylococci. One TGC safety strain which is outfitted in this way is Bacillus subtilis, which is additionally outfitted with listeriolysin from L. monocytogenes. An example for the outfitting of apathogenic bacteria for the TGC safety strain is indicated by the outfitting of L. innocua with the hly and/or the actA gene from L. monocytogenes in Example 1. Another example is E. coli K12, outfitted with the invasin gene (inv) from Yersinia pseudotuberculosis.

The TGC method is carried out in the following steps:

a)   Cloning of the TGC (Foreign) DNA:

The TGC method begins by preparation of the L. monocytogenes strain in the laboratory. The cDNA for the foreign protein to be produced is inserted into a suitable vector. The insertion of the cDNA is done in familiar fashion so that the subsequent transcription and expression in the eukaryotic host is assured. If the protein is to be secreted by the cell, the vectors must contain suitable host cell-specific signal sequences. The vector can be an eukaryotic vector, for example, pCMV of the Clontech Company or pCMD of the Invitrogen Company, both commercially available. As important criteria for selection vectors, these eukaryotic promotors have donor and acceptor sites for the RNA splicing (facultative property), as well as polyadenylation site, for example, from SV40. For multiplication of the DNA, the production of the genetic constructs (hereinafter termed TGC-DNA) can be carried out in E. coli or any other suitable host strain by [familiar?] methods. The TGC-DNA only needs to be brought into the selected bacteria for the

primary cloning and can be transferred afterwards into the selected bacterial TGC safety strain. The transfer into L. monocytogenes can be done with the various well-known methods of gene transfer of isolated DNA (transformation, electroporation, etc.) or by the processes of conjugation and transduction directly or indirectly from bacterium to bacterium.

b)   TGC Safety Strains as Recipients of the TGC-DNA:

Used as the recipient of the TGC-DNA are especially L. monocytogenes host strains - or also other TGC foster parents, representing intracellular vital bacteria like L. monocytogenes (e.g., Yersinia), or those which penetrate to the endosome (e.g., Salmonella), or even otherwise nonpathogenic bacteria which are "outfitted" with additional bacterial factors (e.g., Escherichia coli or L. innocua), fulfilling the following properties, alone or in combination:

(A.1)   they are suitable as receptors of foreign DNA (can be genetically manipulated);

(B.1)   they carry mutations affecting genes vital to the survival of the bacteria in the outer world, i.e., outside a host, such as at rather low temperature (safety-relevant property);

(B.2)   they are attenuated host strains in which a portion of their pathogenicity factors is deleted or inactivated, so that they no longer display the full pathogenicity of the wild type strains (attenuation);

(C.1)   they are "genetic cripples," which because of metabolic defects specifically introduced by the experimenter can only be cultured on certain artificial media, but because of these defects can no longer grow in the cell or especially the entire animal, and thus can no longer reproduce themselves ("endogenous suicide");

(C.2)   they induce their uptake into endosomes and are dissolved in these compartments of the cell (infection via endosomes)

(C.3)   they are taken up by professional phagocytes in phagolysosomes, but can dissolve these cell compartments (i.e., overcome them) (infection via phagolysosomes)

(C.4)   the bacteria carry suicide genes, which are conditionally activated only after penetrating into the host cell, so that the bacteria kill themselves ("exogenous suicide")

(D.1)   they are to be eliminated by antibiotic treatment of the subsequent animal host (killing by antibiosis).

Item A.1 is a general property of bacteria, without which none of the mentioned genetic manipulations would be possible.

Items B.1 and B.2 cover alterations which make the use of the bacteria more safe. Bacteria with these alterations can no longer reproduce when let out into the environment, or they are attenuated (B.1) and have a lower pathogenic potential (B.2). The altering of bacteria per subitem B.2 also has influence on the releasing of the foreign DNA into the cell (see Items C.2 + C.3).

Items C.1 - C.4 involve genetic alterations of bacteria which critically determine the releasing of the foreign DNA into the animal cell. Items C.3 - C.4 indicate infection routes which have been identified, for bacteria summarized hereafter among the examples, as a way of transmitting the foreign DNA into the cytosol of the animal cell.

The intervention mentioned under (D.1) enables the targeted killing of the bacteria. In this way, one accomplishes a releasing of the foreign DNA from the bacteria, but the antibiotic therapy is also a safety-relevant aspect.

The releasing of the recombinant DNA into the cell becomes possible only through the alterations and interventions per C.1 - C.4, and also B.2 and D.1.

Strains with these properties (individually or in combination) are termed TGC safety strains.

c)   Optimization of the TGC Foster Parent for the Target Organ of the TGC Method:

The TGC-DNA which codes for the foreign protein to be produced is transferred into the TGC safety strain by transformation, conjugation, or transduction. The strains thus obtained are subsequently termed TGC foster parent. The foster parent furnishes (feeds) the DNA to the TGC host and thereby induces the somatic transgenesis. In order for the desired foreign protein to be optimally expressed during the TGC method, the gene should advantageously be under the control of promotors and other regulatory acting sequences which derive from the previously selected target organ of the TGC method or which are optimized for the target organ, such as udder-specific promotors and secretion signals.

d)   Infection of the Host Organism with the TGC Foster Parent:

By culturing the TGC foster parent, it is multiplied in vitro in a culture medium and prepared to carry out the TGC method in a selected host organism. As an alternative, the TGC foster parent can also be multiplied in the host organism (man or animal, hereinafter called the TGC host), by in vivo culturing. As preparation for the infection, the TGC foster parent is taken up in a nonbactericidal solution, a buffer, or another physiological fluid adapted to the TGC host. The fluid is administered to the TGC host, for example, the lactating mammal, when the udder is to be made somatically transgenic. This can be done either perorally by drinking the fluid or by administering through a stomach probe, the anus, or another bodily orifice. As an alternative, one can consider administering the TGC foster parent by injection, which is intravenous, intramuscular directly into the target organ, or preferably intraperitoneal. As another alternative, the infection can be done by producing an aerosol and subsequent inhalation of the droplets.

The TGC host (man or farm animal: cow, horse, goat, sheep, pig, rabbit, poultry, etc.) can be infected several times with identical or heterologous transgenes. By repeated infection with different DNA, coding for several enzymes of a biosynthesis pathway, for example, one can establish entire enzyme cascades in the TGC host. In this way, one can also accomplish the biochemical expression of multigenic proteins.

e)   Organ and Cell Specificity of the Infection:

The further route of the TGC foster parent in the organism is determined at first by the natural infection pathway. The TGC foster parent reaches the target organ on the typical route controlled by the particular bacterium. In the case of L. monocytogenes, if the TGC foster parent is carrying genetically unaltered internalins, the udder is one of the target organs. Genetically altered internalins enable the infection of different organ systems. According to its infection cycle, the TGC foster parent penetrates into the cells and appears in the cytoplasm. Since it is genetically defective, the TGC foster parent cannot further multiply there, and it undergoes "endogenous suicide" (see above, C.1 under heading (b)). When infecting the cell, the TGC foster parent introduces the host-foreign TGC-DNA in its package into the cell. However, the transmission of the foreign DNA into the cell can also be triggered by "exogenous suicide" (see above, C.4 under heading (b)) or by "killing" the bacteria with targeted antibiosis (see above, C.3 under heading (b)). In these three cases, the bacterial cell carrying the foreign DNA dies inside the animal cell and thus the foreign DNA is released into the cytoplasm. Finally, the transmission of the foreign DNA to the animal cell can also be accomplished by targeted infection of the cells with no lysis of the endosomes. In this case, the foreign DNA of the animal cell becomes accessible by lysis of the bacteria inside the endosomes.
In each of the mentioned cases, the DNA transferred to the cell is now available as a template for production of the desired foreign protein. But the nucleic acid can also have a direct therapeutic effect, for example, by generating an anti-sense RNA. The so manipulated cells, tissue or organs become somatically transgenic in the course of the infection.

f)   L. monocytogenes Induced Protein Production in the Milk of Mammals:

After carrying out the TGC method - for example, with TGC foster parents such as L. monocytogenes, but also other intracellular vital bacteria (e.g., Yersinia), or those penetrating into the endosome (e.g., Salmonella) or otherwise nonpathogenic bacteria outfitted with additional bacterial factors (e.g., Escherichia coli or L. innocua) - the protein is formed in the lactating cell and secreted into the milk with the other products of the cell. If several animals are made somatically transgenic with various foreign DNA in the TGC method, the different proteins can be produced separately from each other by collecting the milk of each individual TGC host.

Thanks to the properties of the TGC foster parent, no L. monocytogenes (TGC foster parents, i.e., bacteria) occur in the milk. If, however, such is the case, the bacteria can be eliminated by all methods familiar to the technician, for example, by treatment with antibiotics. The animals (or also the human) after undergoing the "targeted genetic conditioning" (TGC) are free of viable, genetically altered organisms and thus undergo no further safety determinations. The TGC host passes on the genetic information brought in through the TGC method to the next generation of cells as part of ordinary cell division. But the information is not transmitted to progeny of the TGC host, since the TGC-DNA is not contained in the germ tract of the TGC host. The bypassing (i.e., omitting) of genetic manipulation of the germ tract of the overall organism and the targeted protein production in the predetermined organ or tissue of the animal host (animal and man) constitutes the innovative and new aspect of the invented method.

g)   Infections of Tissues by L. monocytogenes

Blood is a tissue whose genetic alteration by the TGC method according to the invention will be described as an example. The cells of blood are especially suitable for the TGC method. The infection of blood cells can be done outside the body. The somatic transgenesis of the cells can likewise be verified outside of the host. In the case of attenuated auxotrophic bacteria, the substances essential to the growth of the cells - diaminopimelic acid serves here as the example of auxotrophism - can be supplied to the medium and thus control the period of viability of the bacteria according to the goal of the experiment. By subsequent lysis of the animal cells, one can then check whether the intracellular bacteria are still vital.

The transfected cells, containing a precisely known quantity of vital bacteria, are finally used for reimplantation into the recipient organism. In certain cases, these quantities of bacteria can be so large that other organs are infected in the organism. In other cases, the transgenesis is deliberately confined to the tissue blood by eliminating vital bacteria in vitro before reimplantation into the TGC host.

The reimplantation and associated dissemination of the transgenic cells with or without vital bacteria enables the somatic gene therapy of cells in the host, which in this case can also be a human.

But the TGC method also enables producing extracorporal proteins. For this, the TGC foster parents are injected into poultry eggs. Suitable techniques for this in the production of vaccines for viral pathogens are already prior art. In the course of the incubation of the eggs, the cells in the egg are infected, become somatically transgenic, and then produce the foreign protein. The foreign protein can be purified from the egg by familiar techniques. In this form of the TGC method, the TGC foster parent remains controllable in all phases of the application under laboratory conditions. The quantity of the protein being produced depends only on the injection of an appropriately large number of eggs. '

h)   Use of the TGC Method for Somatic Gene Therapy:

As of yet there is no established form of somatic gene therapy. At present, the nucleic acid used for the transfection is protected in the interior of viruses or packaged in liposomes against influences from the outer world.

Viruses have the drawback of having only a limited uptake capacity and when the infection dose is high they can be expected to develop their full cytopathic effects [32a]. They induce immune reactions and can thus be attacked and destroyed themselves. Some viruses are inactivated by serum and then become unusable for the gene therapy. Especially noteworthy here is the multiple administering of viruses for gene therapy, in the course of which the immune response of the host is encountered. Lastly, the formation of a targeted defense against the viruses has turned into a major problem when using viruses for gene therapy.

When using liposomes, one must consider the toxic effect of the lipids, which trigger inflammatory reactions.

In the case of in vivo therapy, there are still considerable deficiencies in the way of applying the gene transfer systems used thus far. This form of therapy requires [32b]:

(i)   resistance of the vector to breakdown after in vivo administration in the body,

(ii)   tissue specificity, i.e., targeting of the tissue (organ) being treated, and

(iii)   safety, by which is meant no harm to organs which are not being treated [32b].

The bacteria listed in this patent application for use as a vehicle in gene transport and gene transfer are ideally suited to gene transfer. The bacteria are optimally adapted to their particular host and can survive in it for a sufficient time without external intervention, such as antibiotic therapy. They induce specific diseases by defined infection pathways and some of them exhibit a pronounced organotropism. They can take up substantial quantities of foreign DNA (e.g., naturally occurring plasmids have sizes of several 100 kilobases), so that not just c-DNA, but even larger regions of a chromosome can be transmitted. Finally, they can be used safely, especially in the case of "cripple bacteria," as have been described above. The genetic defects of the TGC foster parent in combination with the aforesaid antibiotic sensitivity guarantee an efficient elimination of the bacteria after they have performed their task of DNA transfer into the eukaryotic cell.

Example:

We mention here examples of a somatic gene therapy:

-       The treatment of cystic fibrosis (CF): For this the microbe must be administered to the patient being treated by inhalation. The bacterium is preferably a microbe which is transmitted by droplet infection. The bacterium contains the CFTR gene, which can cure the major defect in CF. The bacterium penetrates into the columnar cells of the airway (airway lumen-facing columnar cells) and transfects them with the CFTR-DNA integrated in the TGC vector. The cells become somatically transgenic, and the defect is cured.

-       Somatic gene therapy with the human ß-globulin gene can treat ß-thalassaemia. For this, stem cells of the haematopoetic series are infected ex vivo with a TGC safety strain, which transmits the ß-globulin gene to the stem cell. By treatment of the cells in the cell culture, the infecting bacterium is eliminated and the transgenic cell is prepared for back-transmission to human. This transmission is done by intravenous administration.

-       In the treatment of Hurler's Syndrome, primitive CD34-positive cells of the bone marrow are transfected with the α-L-iduronidase gene. The gene therapy route and the back-transmission of the cells to the patient corresponds to that described in the previous section.

-       In gene therapy for Fanconi's anemia, the gene of the Fanconi anemia complementation group C (FACC) is used for the somatic gene therapy. The target cells for infection with the TGC foster parent in this case are once again CD34-positive cells of the bone marrow.

i)   Detection of the Result of the TGC Method

The DNA transfer becomes visible in the mouse already within the first 24 hours, i.e., long before a specific immune response against the bacterium could arise. This was demonstrated by the production of ß-galactosidase or the fluorescing protein EGFP in cell cultures within 24 hours. The "mitogenic effect of the bacteria" additionally occurring as part of the infection favors the establishment of the DNA in the TGC cell and is therefore desirable and advantageous to the success of the TGC method.

Therefore, in summary, the use of bacteria for somatic gene therapy is more safe than gene therapy with viral systems. The bacterial infection can be directed and locally confined. The growth and thus the florid infection by the bacteria can be prevented by precluding certain bacterial factors. Furthermore, the growth of the bacteria in the eukaryotic cell can be precisely influenced and generally prevented. Finally, the bacterial infection can be ended at any time by the use of antibiotics, i.e., the infection can be effectively limited in place and time.

The invention is described in greater detail hereafter on the example of L. monocytogenes:

Example 1: The Production of TGC Safety Strains

The L. monocytogenes safety strains are produced by targeted genetic alterations of primary pathogenic L. monocytogenes strains. Several echelons of safety are established in parallel. This prevents the vitality or pathogenicity from returning by back-mutations. The mutations involve genes which (1) influence the survival of the bacteria in the cell, (2) diminish the pathogenicity of the bacteria in the TGC host, and (3) prevent the survival of the bacteria in the environment after a possible elimination.

a)   First Echelon of Safety - S afety-Relevant Property: Survival in the Environment (See Above, B.1 Under Heading (b))

The TGC foster parents can be applied to the TGC host either by injection or by peroral administration. In peroral administration, an oversupply of bacteria and thus an elimination of bacteria not taken up by the organism may ensue. So that these eliminated bacteria have no chance of surviving in the environment, the TGC safety strains can contain additional mutations which prevent the growth of the bacteria in the environment.

One example of this is to switch off the cspL gene (cold shock protein of Listeria). This has the consequence that the bacteria can no longer grow at temperatures below 20°C. The growth and the infectiousness at 37°C are not impaired, yet additional modulation is done by simultaneous mutations per a) and b). The cspL gene, which is deleted in the safety strains used according to the invention, is represented in the sequence protocol as SEQ. ID No. 2. A corresponding cspL-deleted strain has been deposited under the designation L. monocytogenes EGD delta cspL1 with the DSM as No. 11883.

The TGC safety strains used according to the invention can only be cultivated on special growth media. The growth temperature must be over 37°C; no growth is possible below 20°C. The bacteria

have a limited pathogenicity and are only able to make their way into limited, narrowly circumscribed areas of the TGC host. This guarantees the safety of the system to man and environment. The TGC foster parents are no longer able to grow outside the artificial media, here, especially in the host cell. This limited intracellular viability is at the same time a prerequisite for the releasing of the TGC-DNA in the host cell and, thus, for the induction of the somatic transgenesis in the TGC method.

b)   Second Echelon of Safety - <u>Attenuation</u>: Reduced Pathogenicity (See Above, B.2 Under Heading (b))

The second echelon of attenuation of the TGC safety strains comprises mutations in the pathogenicity factors. By targeted mutations in defined factors, the pathogenicity of the bacteria is weakened, the induction of apoptosis of the infected host cell prevented, and at the same time the immune response is guided in a desired direction. The mutations restrict the intracellular motility of the bacteria and thus their propagation into secondary cells. In this way, the infection is limited to the selected target cells while retaining the option of treatment with known antibiotics.

For safety reasons, it is desirable to restrict or even prevent the intracellular propagation of the TGC foster parent after the infection. Precise knowledge as to the intracellular life cycle and the motility of the above-mentioned bacteria makes it possible to create defined, stable mutants with reduced ability to infect the TGC host.

For L. monocytogenes, the mutations attenuated in this regard involve, for example, the hly gene with the result of blocking the infection of the first cell. As an example of the elimination of this pathogenicity factor, the strain L. monocytogenes EGD Hly$_{D491A}$ has been deposited and has been given the deposit number DSM 11881.

Another example for reducing the pathogenicity of L. monocytogenes are mutations in the actA gene or the deletion of regions which are necessary for the interaction between actA and the host cell protein VASP, with the result that the intracellular motility is blocked. Finally, there are also mutations of the plcB gene, which take away the ability of the bacteria to expand into a second cell. The deposited strain L. monocytogenes EGD delta actA delta plcB is an example of a double mutation, in which both the actA gene and the plcB gene are switched off. It carries deposit number DSM 11882.

Furthermore, it is also possible to exchange the wild type listeriolysin gene for a mutated allele in L. monocytogenes. The properties of listeriolysin, both to induce an apoptosis in different host cells and to generate a strong T-cell-mediated immune response, are then restricted.

c)   Survival in the Cell: - <u>Endogenous Suicide</u>: Third Echelon of Safety (See Above, C.1 Under Heading (b))

In general, the bacteria attenuated for the TGC method are characterized by defined deletions in the genes which are essential to the biosynthesis of integral bacterial components. The selected auxotrophic bacteria are suitable as TGC foster parent, for as attenuated bacteria they can transport foreign DNA into the cell. But since the bacteria are cut off from essential "growth factors" in the

cells, they spontaneously lyse and thereby release the TGC-DNA in the cell.

As TGC safety strains, one uses L. monocytogenes which are genetically altered so that they still infect the cell, but can no longer multiply in the cell. This is accomplished, for example, by inactivating the dapE gene in L. monocytogenes. Listeria are Gram-positive bacteria, which like the Gram-negative bacteria require meso-diaminopimelic acid (DAP) derivatives for cross-linking of the cell wall. The biosynthesis of diaminopimelic acid is therefore essential to the formation of the bacterial cell wall. DAP-auxotrophic bacteria are subject to spontaneous lysis when this amino acid is no longer provided in the culture medium. The enzymes involved in the synthesis of DAP in the bacterium are not present in mammalian cells. These enzymes are likewise deleted in TGC safety bacteria, or are inactivated by insertions or otherwise. The dapE of L. monocytogenes, which has been inactivated in the safety strains used by the invention, is represented in the sequence protocol as SEQ. ID No. 1. For the genetic manipulation of the dapE gene in L. monocytogenes its sequence had to be determined; the corresponding genes, such as from E. coli, have only around 30% homology with the sequence of SEQ ID No. 1.

The bacteria deprived of this or other genes of the DAP biosynthesis pathway, so-called DAP mutants, cannot grow either inside or outside the host. For this, they need the addition of large quantities of DAP (1 mM) to the growth medium. Without DAP, the bacterium cannot survive either in the TGC host or outside the TGC host. Thus, these DAP mutants offer both safety from a bacterial infection of the TGC host and safety from an infection of other organisms in event of the release of such a strain into the outer world.

An intervention in the genome of Salmonella with corresponding effects (creation of an auxotrophic mutant) involves the deletion (or blocking or mutagenesis) of the aroA gene, which is essential to the synthesis of aromatic amino acids. From the Salmonella inoculation strain (available at the American strain collection under ATCC14028), a mutant can be obtained by genetic manipulation with techniques familiar to the practitioner and given knowledge of the aroA gene (Genebank Reception No. M 10947) that can function as a TGC safety strain similar to the recombinant bacteria described here for Listeria. As for the above-described L. monocytogenes delta dapE strain, the releasing of the foreign DNA occurs by killing of the bacteria after being taken up into the cell. Unlike L. monocytogenes, Salmonella is not able to get into the cytoplasm of the cell. In this case, the releasing of the foreign DNA occurs from the endosomes into the interior of the cell.

Still other attenuated mutations of L. monocytogenes are also known, in which the biosynthesis of nucleic acids, amino acids, sugars or other building blocks of the cell wall are blocked [33 to 35]. The same can also be achieved by mutations in regulatory genes that are essential to the intracellular life cycle of the bacteria. An example of one such gene is phoP of Salmonella typhimurium [36].

The examples given here for L. monocytogenes can be transferred to other intracellularly living bacteria or bacteria that have been turned into intracellular pathogens by outfitting them with pathogenicity factors. This holds in particular for bacteria of the genera Aeromonas, Bartonella, Brucella, Campylobacter, Clostridia, Enterobacteriaceae (especially E. coli, Salmonella, Shigella, Yersinia), Mycobacterium, Renibacterium and Rhodococcus. One TGC safety strain outfitted in

this respect is, for example, Bacillus subtilis, which is additionally outfitted with the listeriolysin from L. monocytogenes.

An important prerequisite for the transmission of DNA itself into "remote" body cells is protecting the DNA on the way to the target cell or the target tissue or the target organ. The ability of intracellularly living bacteria such as L. monocytogenes to propagate intracellularly is an ideal quality for transporting genes to remote cells, deeper tissue and organs. After successful transfer of the TGC-DNA to the target cell, the messenger or TGC foster parent dies, as a result of the attenuation (B.1), the induction of auxotropisms (B.2), endogenous suicide (C.1), infection via endosomes (C.2), infection via phagolysosomes (C.3), exogenous suicide (C.4) or antibiotic therapy (D.1).

Example 2: Release of Foreign DNA in the Animal Cell (Synonym: Tissue, Organ)

a)   Infection via endosomes: Transfer of the expression plasmid without liberating the bacteria from the endosome vesicle (see above, C.2 under heading item (b))

A test was performed to see whether bacteria are able to transmit their plasmid DNA into the cytoplasm of infected host cells, without having to first be liberated from the endosome vesicle. For this, the ability of the L. monocytogenes Dhly mutant which can no longer emerge from the endosome was investigated to function as a transmitting bacterium for the DNA transfer. The foreign DNA being transferred was chosen to be EGFP, a fluorescing protein that was cloned under the control of a CMV promotor. As the yardstick for successful transmission of the foreign DNA - i.e., a yardstick for the transfection of the eukaryotic cell - 10,000 cells were investigated in the FACS scanner after being infected with the particular L. monocytogenes strains for the occurrence of the EGFP-triggered fluorescence. The figure is expressed in Table 1 as a percent of the total number of eukaryotic cells measured. During the experiments, the L. monocytogenes wild type strain EGD served as the positive control. An isogenic, noninvasive ΔDInlAB strain was also tested. The information obtained with these bacteria is of general validity and can be transferred to other bacteria.

The findings are summarized in Table 1 and show that the Dhly mutant is just as efficient in regard to the DNA transfer from the bacterium to the eukaryotic cell as is the L. monocytogenes wild type strain. The L. monocytogenes DInlAB strain is not suitable as a vehicle for the DNA transfer into the cells indicated here (PtK2), or it is substantially less suitable (Hep-2). The experiments furthermore show that the active uptake of the bacteria by the eukaryotic cell (in this case, not professional phagocytes) is the prerequisite for the transfection of the cells. The attachment of the bacteria is accomplished by the interaction between bacterial internalins (InlA and/or InlB) and the receptors of the animal cells. The experiments of the following example show that internalins are not necessary for uptake of bacteria into professional phagocytes.

Cell Line	Origin	L. monocytogenes Strain	Transfected Cells in %

PtK2	Kangaroo rat kidney	Wild type EGD	1.71
		Δhly	1.78
		ΔinlAB	0
Hep-2	human larynx carcinoma	Wild type EGD	4.58
		Δhly	4.31
		ΔinlAB	0.24

b)   Infection via phagolysosomes: Outfitting of nonpathogenic strains into a TGC safety strain: (see above, C.3 under heading item (b))

The example presented hereafter for L. innocua is exemplary and can be transferred to other nonpathogenic bacteria (e.g., Escherichia coli). The steps to be taken in the genetic outfitting of such bacteria correspond to those mentioned here for L. innocua.

A nonpathogenic L. innocua strain (Serovar 6a) was "outfitted" with the pathogenicity factors of listeriolysin and ActA from Listeria monocytogenes. In order to regulate the expression of these genes, the transcription factor with in transpositive effect (PrfA) was cloned in the genetically manipulated L. innocua strain as a third gene. The presence of PrfA makes the expression of the virulence genes dependent on the growth temperature. Since this recombinant L. innocua strain possesses no internalins, i.e., it is not invasive in itself, it cannot penetrate into the aforementioned cells (Ptk2; Hep-2). If the experimenter wishes to also be able to infect such cells, the bacteria must be further outfitted with the internalins InlA and/or InlB. The experiments of the present example show that these bacterial products (internalins) are not necessary for the uptake of L. innocua (hly+; actA+) strain by professional phagocytes. After its phagocytosis, the L. innocua strain (hly+; actA+) uses the protein listeriolysin for lysis of the phagolysosomes of the professional phagocytes. In the electron microscope image one recognizes that the genetically manipulated L. innocua (hly+; actA+) strain shows up in the cytoplasm of the professional phagocytes. The wild type strain L. innocua Serovar 6a, on the other hand, is killed in the phagolysosome and does not appear in the cell's cytoplasm. The expression of the ActA protein allows the L. innocua (hly+; actA+) strain an intracellular movement dependent on the actin cytoskeleton, which in the EM image resembles the motion of the L. monocytogenes strains. Due to the absence of additional genes, such as the plcB gene, the L. innocua (hly+; actA+) strain mentioned here cannot spread to neighboring cells. This specific change in the infectiousness has already been described for recombinant L. monocytogenes ΔplcB strains.

The targeted selection of genes, here, hly and actA, and their transformation into nonpathogenic bacteria, transmits the selected L. monocytogenes properties to nonpathogenic bacteria. The liberation of the bacteria from the "deadly" phagolysosome is the prerequisite for the transfer of

foreign DNA into the infected cells. The DNA being transmitted for the reprogramming of the animal cells is integrated into constructs, as have already been described above for attenuated L. monocytogenes bacteria - which can already be employed as such by the invention. The releasing of the genetic information occurs, according to the invention, by (i) creation of auxogenic mutants (deletion of endogenous, vitally essential genes), by (ii) introduction of "suicide genes," (iii) by induced uptake in endosomes and killing therein, or (iv) by an antibiotic therapy defined in terms of time and adapted to the killing of the bacteria in a target organ or tissue.

The experiments of this example show in exemplary manner how bacteria not naturally pathogenic can be subsequently "outfitted." By outfitting with definite bacterial factors (here, a gene, i.e., properties of naturally invasive bacteria), bacteria which are otherwise primarily unsuitable for the TGC technique can be manipulated and controlled by the experimenter so that they can be used for a controlled infection and transmission of DNA into the animal cell (synonym: tissue, organ, whole animal, man).

c)   Release by exogenous suicide: Cloning in of suicide genes: (see above, C.4 under heading item (b))

Suicide genes, which are activated after penetrating into the host cell and result in the death of the bacteria, can be supplied to the bacteria in the form of lysis genes from bacteriophages, for example, with the S-gene of the bacteriophage Lambda or its analogues [37], or with killer genes from plasmids [38]. These genes are under the control of an intracellularly inducible promotor (for example, pagC promotor from Salmonella [38]).

d)   Releasing by antibiotic therapy: Targeted release of foreign DNA in the lung after droplet infection of Listeria monocytogenes (see above, D.1 under heading item (b))

The infection with the bacteria was done by the method of "body plethysmography in spontaneously breathing mice" per R. Vijayaraghavan [Arch. Toxicol. 67: 478-490 (1993)]. In the experiment, the mice were individually exposed for half an hour in an inhalation chamber to an aerosol of a milliliter bacterial suspension, containing a total of 5000 bacteria. This number of bacteria corresponds to a LD50 dose for intraperitoneal administration of bacteria. In order to track the course of the infection in real time, the bacteria were again transformed with an EGFP gene construct. The path of the bacteria in the animal model was traced by fluorescence analysis of the EGFP protein formed in the tissue. According to this, the bacteria penetrate within half an hour to the columnar and endothelial cells of the airway. At this time, no bacteria are to be found in other tissue or organs of the infected animal, such as spleen, liver, brain. The infection remains exclusively confined to the lungs for up to 18 hours. Only after 24 hours are other organs also attacked.

The experiment shows that the propagation of the bacteria after droplet infection can be confined to the primary organ by manipulating the viability. Two ways of accomplishing this are the use of attenuated mutants (e.g., deleted in the "propagation gene" ActA) and/or the destruction of the bacteria by administering antibiotics at a time dictated by the experimenter, i.e., in an organ dictated by the experimenter.

Example 3: Description of the TGC Vectors

TGC vectors are episomal DNA, for example, plasmids with little ability to take up foreign DNA (pMB descendants, sufficient for individual genes), or with greater DNA uptake capability (as for P1 or F plasmids), in order to create the somatic transgenesis for complex biosynthesis pathways.

In all instances, plasmids are involved which replicate in the hosts for the genetic alteration and for cultivation of bacteria used for the TGC method. An example for an intermediate host in which genetic building blocks can be constructed are E. coli or other bacteria normally used in recombinant DNA technology. One suitable TGC safety strain is L. monocytogenes or the other above-mentioned bacteria which function as TGC foster parent. In order to fulfill this condition, the plasmids receive the host-specific plasmid replicon sequences. In the creation of recombinant DNA, one must distinguish between transformed and "naked" host cells. The customary antibiotic resistance genes can be used as selection principles for this.

Example 4: Transformation of L. monocytogenes Safety Strains into TGC Foster Parents

The transformation of L. monocytogenes occurs by a modified protocol of Park and Stewart [40].

For this, bacteria up to an optical density of $OD_{600} = 0.2$ are used. Ampicillin (10 µg/ml) and 1 mM glycine is added to the culture medium. A further multiplication up to an OD_{600} of 0.8 to 1.0 then ensues. The cells are harvested by centrifugation and taken up in 1/250 vol. cold electroporation buffer (1 mM hepes, pH 7.10; 0.5 M sucrose). The bacteria are washed up to 4 times as preparation for the electroporation.

For the electroporation, 50 µl of the prepared cells are placed in an electroporation vessel, and electroporation is done at 10 kV/cm, 400 Ohm, 25 µF, using around 1 µg of DNA.

After this, the cells are placed immediately on ice and taken up in 10-fold BHI medium and incubated for 2 hours at 37°C under careful shaking. After this time, the cells are plated out and inoculated at the desired temperature. The efficiency of the transformation by this method is 10^4 to 10^5 transformants per µg of plasmid DNA used.

Example 5: Description of the Cultivation of TGC Foster Parents For Use in the TGC Method

Listeria were preferably grown in the brain-heart infusion broth, for example, BHI of the Difco Company. Alternatively and for special applications (radioactive labeling of listerial proteins), the bacteria can be grown in tryptic soy broth (TSB) or in Listeria minimal medium (LMM) [36]. The bacteria are centrifuged and washed repeatedly in suitable transfer medium, for example, a buffer containing bicarbonate.

Such prepared bacteria can be kept for at least six months at -80°C by adding 15% glycerin solution before being used in the TGC method.

Example 6: TGC Method - Feeding of the TGC Foster Parents

To begin the TGC method, the animals are deprived of drinking water for several hours. The (TGC foster parents: TGC-DNA in the desired TGC strain) are taken up in a suitable concentration of a buffer containing bicarbonate and administered to the animals orally, by inhalation or by injection (parenteral, intramuscular, intraperitoneal, or directly into the desired target organ). The nature of the application depends on the physiological infection pathway of the corresponding TGC foster parent. The choice of the microbe being used as TGC safety strain depends on the target organ and will be made according to the infection pathway and according to the organotropism of the particular bacterium. The dose of bacteria is chosen such that the desired organotropic incorporation of the TGC foster parent is achieved. The quantity and the type of application of the bacteria will depend on the particular bacterium, but also on the host and the target organ (also see Example 2).

Example 7: Performance of the Somatic Gene Therapy

Examples of a somatic gene therapy given here are:

-       The treatment of cystic fibrosis (CF): For this the microbe must be administered to the patient being treated by inhalation. The bacterium is preferably a microbe which is transmitted by droplet infection. The bacterium contains the CFTR gene, which can cure the major defect in CF. The bacterium penetrates into the columnar cells of the airway (airway lumen-facing columnar cells) and transfects them with the CFTR-DNA integrated in the TGC vector. The cells become somatically transgenic, and the defect is cured.

-       Somatic gene therapy with the human ß-globulin gene can treat ß-thalassaemia. For this, stem cells of the haematopoetic series are infected ex vivo with a TGC safety strain, which transmits the ß-globulin gene to the stem cell. By treatment of the cells in the cell culture, the infecting bacterium is eliminated and the transgenic cell is prepared for back-transmission to human. This transmission is done by intravenous administration.

-       In the treatment of Hurler's Syndrome, primitive CD34-positive cells of the bone marrow are transfected with the α-L-iduronidase gene. The gene therapy route and the back-transmission of the cells to the patient corresponds to that described in the previous section.

-       In gene therapy for Fanconi's anemia, the gene of the Fanconi anemia complementation group C (FACC) is used for the somatic gene therapy. The target cells for infection with the TGC foster parent in this case are once again CD34-positive cells of the bone marrow.

Example 8: Checking for the Result of the Induced Somatic Transgenesis

After the transfer of the TGC-DNA into the TGC host, the outcome of the TGC method is to be detected. For this, immunological assays of the gene product (protein) are suitable, using immunoassays such as the ELISA, the Immunoblot, or other known assays based on an antigen-antibody reaction. T-cell responses can be called up in special assays and will always be used when the antigen is a substance which is identified by immune responses mediated by MHC Class I.

If the protein produced is an enzyme, its biological activity can occur in the form of enzymatic activity testing. If the protein has in addition a biological activity, the performance capability of the protein formed will be called up by biological assays.

For proteins which induce a passive or active immunization of the TGC host, the protection will be tested against the triggering agent. For example, it may involve the prevention of the colonization, the infection (or the apparent illness) of the experimental animal after being loaded with the pathogenic organism (bacterium or virus).

Example 9: Harvesting of the Protein

The obtaining of the protein produced is done by techniques which are familiar to everyone engaged in agriculture:

- if the TGC host is a cow or another lactating farm animal and the infected organ is the udder, the familiar techniques of milking will be employed;

- if poultry such as hens are used as the TGC host, the eggs will be gathered and subjected to protein purification;

- the processing of proteins from organs whose products are not delivered to the outside is done by harvesting the corresponding organ, which will generally require slaughtering, as in the case of fish;

- if blood is the somatically transgenic tissue, the desired product will be obtained after puncturing a vein from the blood or its cells and purified with methods familiar to the technician.

Example 10: Purifying of the Protein

A preliminary purification of the protein being produced is done by separation methods, using primary physical or physicochemical methods well known to the technician. These include precipitation of proteins with salts (for example, ammonium sulfate), with acids (for example, trichloracetic acid), or under the action of heat or cold.

A rough separation is also achieved by means of column chromatography. All methods used here depend very greatly on the primary media in which the particular protein is accumulated. Thus, for example, for the processing of milk or eggs, many methods are familiar in industry, which can also be applied to the invention described here. The same holds for the processing of blood as a somatically transgenic tissue. Here, one can make use of the experience in transfusion medicine, especially the processing and purification of blood clotting factors.

Example 11: Cleaning of the Protein

For the final cleaning of the protein, one can employ all methods used for conventional cleaning of proteins. They include

-       cleaning by means of affinity chromatography, for example, using the receptor-ligand interaction;

-       the presentation of fusion proteins with so-called "tags," which can be used for specific interaction with a chromatography matrix (for example, poly-histidine tag and nickel-column chromatography; the streptavidine-biotin technology of affinity purification). The tags can be removed once again by properly introducing an appropriate protease intersection with subsequent protease digestion;

-       cleaning by means of specific antibodies (immune affinity chromatography);

-       the use of natural affinities between the target protein and other proteins, carbohydrates, or other binding partners, as in the case of toxin A of Clostridium difficile, its binding ability to thyroglobin at 4°C and its subsequent elution by raising the temperature to 37°C.

Example 12: Production of TGC Proteins:

The list of proteins which can be produced by the TGC method is theoretically unlimited and encompasses primarily the area of the hormones, regulation factors, enzymes, enzyme inhibitors, human monoclonal antibodies, as well as the production of surface proteins of pathogenic microorganisms or viral envelope proteins for nonhazardous production of diagnostic tests and tolerable vaccines. They include both mass articles such as human serum albumen and also proteins which are used in small amounts, such as hirudin, blood clotting factors, antigens for tumor prevention and for active immunization (for example, papilloma antigen), as well as antibodies for passive immunization.

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[in English, except the following]

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Patent Claims:

1. TGC method for induction of a targeted somatic transgenesis in an animal host, characterized in that bacteria with a foreign DNA integrated in an episomal vector and under the control of eukaryotic, regulatory elements for later transcription and expression release the foreign genes in the host upon infection of an entire organism and bring about the transcription and expression of foreign DNA and/or foreign protein therein.

2. TGC method per Claim 1, characterized in that the bacteria release foreign genes upon infection of an organ by targeted perfusion or into culture and thereby accomplish the transcription and expression of foreign nucleic acid and/or foreign protein in the organ.

3. TGC method per Claim 1, characterized in that the bacteria release foreign genes upon infection of an animal tissue and thereby accomplish the transcription and expression of foreign nucleic acid and/or foreign protein in the tissue.

4. TGC method per Claim 1, characterized in that the bacteria release foreign genes upon infection of a mixture of cells, or an individual cell line and thereby accomplish the transcription and expression of foreign nucleic acid and/or foreign protein in the individual cells of the mixture or in the cell line.

5. Method per Claims 1 to 4, characterized in that the foreign DNA introduced by the bacterial infection into the host organism causes the formation there of a protein foreign to or missing from the host organism or increases, reduces or prevents the formation of a protein or the action of a nucleic acid in the host organism by the formation of single or double-strand nucleic acid.

6. Method per Claim 5, characterized in that the foreign DNA introduced by the bacterial infection into the host organism is used

   a) for somatic gene therapy or

   b) for immunological protection against microbial pathogens or

   c) for immunological protection against tumor diseases

and has a preventive or therapeutic effect.

7. Method per Claims 1 to 6, characterized in that bacteria are used from the genera Aeromonas, Bartonella, Brucella, Campylobacter, Clostridia, Enterobacteriaceae, Legionella, Listeria, Mycobacterium, Renibacterium, Rhodococcus or bacteria from genera which are genetically or biochemically related to them, which are intracellularly viable in the eukaryotic host organism.

8. Method per Claim 7, characterized in that the bacteria are weakened or strengthened by selection and genetic manipulation of endogenous bacterial pathogenicity-associated genes, preferably in their in vivo pathogenicity, such that the bacteria penetrate

a)   into definite organs of the overall organism,

b)   into certain tissues of the host organism or

c)   into certain compartments of cells

and liberate the foreign DNA there.

9.   Method per Claim 8, characterized in that the manipulated bacteria are Listeria.

10.   Method per Claim 9, characterized in that the manipulated bacteria are Listeria with deposition numbers DSM 11 881 and DSM 11 882.

11.   Method per Claims 9 and 10, characterized in that the genes in the bacteria with SEQ ID No. 1 and SEQ ID No. 2 as mentioned in the sequence protocol or genes agreeing with these by at least 35% of the nucleotide positions are genetically mutated, deleted or blocked.

12.   Bacterial strain for the TGC method for induction of a targeted, somatic transgenesis, characterized in that the foreign DNA integrated in the vector in it and prepared for later transcription and expression is under the control of regulatory elements which derive from the target organ being infected or are oriented to this target organ.

13.   Bacterial strain per Claim 12, characterized in that it is mutated into a safety strain such that it can no longer adapt its growth to the environmental conditions on account of a mutation in a gene (cspl mutant DSM 11 883) and/or is genetically altered by an auxotrophic mutation per SEQ ID No. 1 and/or by a mutation in the sense of endogenous attenuation (strains DSM 11 881 and 11 882) and/or by additional outfitting with an exogenous suicide gene.

14.   Bacterial strain per Claim 13, characterized in that it is mutated into a safety strain in that

a)   the cspl gene per sequence protocol SEQ ID No. 2 or a gene agreeing with it by at least 35% of the nucleotide positions is mutated or blocked or

b)   the cspl gene is deleted (strain DSM 11883),

c)   the dapE gene per sequence protocol SEQ ID No. 1 or a gene agreeing with it by at least 35% of the nucleotide positions is deleted or blocked or

d)   the actA gene and/or the plcB gene and/or the hly gene or other genes involved in the virulence are mutated, deleted or blocked.

15.   Method per Claim 8, characterized in that the manipulated bacteria are Salmonella, especially Salmonella of the strain with deposition number ATCC14028 or descendants of this strain, which are genetically altered per Claim 14.

16.   Method per Claim 15, characterized in that the bacteria are auxotrophic by a mutation in the aroA gene, deposited in the gene bank sequence M 10947.

17.   Method per Claim 8, characterized in that the genetically manipulated bacteria are apathogenic Listeria, apathogenic or facultatively pathogenic Enterobacteriaceae, or other apathogenic bacteria.

18.   Method for the transfection of animal cells with foreign DNA, characterized in that the bacteria, as carrier of the foreign DNA in the cytoplasm,

a)   are not viable on account of an auxotrophic mutation;

b)   are not viable on account of a foreign suicide gene;

c)   penetrate into the endosomes of the cells, but cannot leave this compartment and are lysed there;

d)   are taken up into phagolysosomes, lyse these compartments, and penetrate into the cytoplasm; and

e)   are destroyed by treatment with antibiotics

and thereby release the foreign DNA.

19.   Method for production of a predetermined foreign protein, characterized in that a selected cell, a selected tissue, or an organ is deliberately bacterially infected and the formation of the predetermined protein is initiated there and subsequently the foreign protein is isolated and purified from the cell, the tissue or the organ.

20.   Method per Claim 20 [sic], characterized in that the expression of foreign protein is induced in the udder of milk-producing animals or in eggs of poultry or in the blood or other tissues of farm animals by infection with bacteria.

21.   Transgenic farm animal, characterized in that all the cells of its organism or the cells of one or more of its tissue or organs are genetically altered by application of the method per Claim 1.

22.   Method for induction of a somatic transgenesis per Claim 3, characterized in that the somatically transgenic tissue is reimplanted in an entire organism and the living entire organism becomes somatically transgenic in this manner.